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Do ovarian steroid hormones control the resumption of embryonic growth following the period of diapause in roe deer (*Capreolus capreolus*)?

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Abstract

Embryonic diapause in the European roe deer comprises a period of five months in autumn in which embryonic development is extremely decelerated. The molecular mechanisms governing the developmental resumption following diapause are not known to date. We investigated the role of steroid hormones in controlling the resumption of embryonic growth until implantation. Our focus was to determine the concentration of progesterone and estrogens between September and January. In plasma and endometrial tissue samples from 145 females shot during regular huntings, progesterone, estradiol-17β and total estrogens were determined. The mean plasma concentrations of progesterone (5.3 ± 0.2 ng/ml, mean ± SE), estradiol-17β (25.0 ± 2.5 pg/ml) and total estrogens (22.6 ± 2.6 pg/ml) remained constant through the different embryonic developmental stages of the blastocyst, elongation and organogenesis. The endometrial concentrations of progesterone (61.3 ± 5.9 versus 16.6 ± 4.0 ng/g, $p<0.05$) and estradiol-17β (293.5 ± 108.1 versus 150.0 ± 48.8 pg/g, respectively, $p<0.05$) were higher during diapause and elongation compared to organogenesis following implantation. We therefore conclude that ovarian steroid hormones do not play a determining role in resumption of embryonic growth following the period of diapause in the roe deer.

Progesterone; estradiol-17β; oestrogens; diapause; roe deer

Zusammenfassung

Die embryonale Diapause beim europäischen Rehen umfasst einen Zeitraum von fünf Monaten im Herbst, in denen die embryonale Entwicklung extrem verlangsamt ist. Die molekularen Mechanismen, die die Wiederaufnahme der Entwicklung nach der Diapause bestimmen, sind bisher nicht bekannt. Wir untersuchten die Rolle, die die Steroidhormone bei der Kontrolle der Wiederaufnahme des embryonalen Wachstums bis zur Implantation spielen. Dabei lag unser Fokus in der Bestimmung der Konzentration von Progesteron und Östrogene zwischen September und Januar im Blutplasma und im endometrialen Gewebe. Es wurden Progesteron, Estradiol-17β und Gesamtöstrogene aus Plasma- und Endometriumproben von 145 Rehgeissen bestimmt, die während der regulären Jagd geschossen wurden. Die Plasmakonzentration von Progesteron (5,3 ± 0,2 ng / ml, Mittelwert ± SE), Estradiol-17β (25,0 ± 2,5 pg / ml) und Gesamtöstrogenen (22,6 ± 2,6 pg / ml) blieben konstant während den verschiedenen embryonalen Entwicklungsstadien von Blastozyste, Elongation und Organogenese. Die Konzentrationen im Endometrium von Progesteron (61,3 ± 5,9 versus 16,6 ± 4,0 ng / g, $p < 0,05$) und Estradiol-17ß (293,5 ± 108,1 versus 150,0 ± 48,8 pg / g, $p < 0,05$) waren höher während der Diapause und Elongation im Vergleich zum Stadium der Organogenese nach der Implantation. Wir folgern daher heraus, dass ovarielle Steroidhormone keine entscheidende Rolle bei der Wiederaufnahme des embryonalen Wachstums nach der Diapause im Reh spielen.

Stichwörter: Progesteron; Estradiol-17β; Östrogene; Diapause; Reh

Do ovarian steroid hormones control the resumption of embryonic growth following the period of diapause in roe deer (Capreolus capreolus)?

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Highlights

- Roe deer samples were collected during huntings from September - January
- Plasma and endometrial progesterone and estrogens were determined
- Plasma steroids remained constant during diapause and elongation
- Endometrial steroids diminished at organogenesis following implantation
- Sex steroids do not drive resumption from diapause in roe deer

Abbreviations

- **CL:** corpora lutea
- **P4:** progesterone
- **E2:** estradiol-17β
- **E_{tot}:** total estrogens
- **CRL:** crown-rump length

1. Introduction

The European roe deer is a monoestric ruminant species (Schams et al., 1980). During the rut in mid-July to mid-August, females (does) ovulate and mating take place (Bischoff, 1854; Ziegler, 1843). Ovulation results in the formation of one corpus luteum (CL) or several corpora lutea, which secrete progesterone (P4) for the following five months (Short and Hay, 1966), irrespective of the pregnancy status (Hoffmann et al., 1978). These five months after the rut season correspond to the time period where the roe deer blastocyst undergoes an obligate diapause (Bischoff, 1854). The phenomenon of embryonic diapause is widespread across the animal kingdom and occurs among multiple mammalian taxa, although some species display embryonic diapause, while closely related other ones do not (Lopes et al., 2004; Mead, 1993). It describes the temporary delay or arrest of development occurring when the embryo is at the blastocyst stage. The embryo persists in the uterus in a reversible quiescent state that lasts from days to almost a year. The roe deer is the only known artiodactyla exhibiting diapause. During the period of diapause in roe deer, embryonic growth is greatly diminished, although mitotic activity is still present at a very low level (Lengwinat and Meyer, 1996). To allow fawns to be born in May-June, a normal growth velocity is resumed in December/January, resulting in rapid embryo elongation, subsequent implantation and epitheliochorial placentation (Bischoff, 1854).

Embryo elongation is a common feature among all artiodactyla and occurs prior to implantation. While the development to the blastocyst stage seems to be rather autonomous and is possible under *in vitro* conditions, the development beyond including embryo elongation is not. It is a critical step in development which to date can only be achieved by support of an appropriate maternal environment. In many artiodactyla, embryo elongation coincides with the embryo`s capacity to prevent the maternal return to cyclicity. Continued luteal progestin production is critical for the maintenance of a growth promoting uterine environment receptive for embryonic signals. Embryonic signalling and maternal recognition of pregnancy are therefore both decisive for the prolongation of the luteal function in order to interrupt cyclicity and to maintain a progestational endometrial state.

The endometrial receptivity for embryo implantation is only given within a short period of time. Historically, this “window of implantation” has been described as the receptive state of the endometrium. In a temporally specific process, the endometrium is primed

by exposure to both estradiol-17β and progesterone of ovarian origin (Finn and Martin, 1972). Adequate remodelling of the uterus also seems to be at least partly dependent on embryo-maternal interactions during the pre-implantation period (reviewed in Gandolfi et al. (1992) and Sponchiado et al. (2017). Hereby, also paracrine actions of the steroid hormones derived from the embryo and/or endometrium as is pigs are responsible for the modification of the uterine epithelium (Young, 2013), which finally allow attachment (Bazer, 2013).

In suidae, the elongated embryo secrets estrogens as anti-luteolysin (Bazer and Thatcher, 1977; Flint et al., 1979; Geisert et al., 1982b). In ruminants, the best known anti-luteolytic signalling factor of embryonic origin is interferon-τ (IFNτ). Both compounds are discussed to promote embryo proliferation in an auto-/paracrine manner. The roe deer is the only ruminant where neither a luteotropic nor an anti-luteolytic signal, such as IFNτ, is known to date (Flint et al., 1994). By displaying a monoestric behaviour, the embryo does not seem to be in need to overcome luteal regression. However, the mechanisms governing embryo growth and developmental velocity in roe deer remain unclear to date.

In diapausing carnivores and marsupials, the growth arrest of the blastocyst is mediated by a non-functional CL and a quiescent uterus (Mead, 1989, 1993; Renfree, 1981). In both species, a luteotropic signal secreted by the embryo during the time of embryo reactivation is not known to exist. However, it is only after the stimulation of the luteostatic CL by GnRH through the hypothalamic-pituitary-ovarian-axis upon changes in photoperiod or the lack of lactational prolactin inhibition that P4 rises and embryonic development continues. Thereupon, the carnivore and marsupial embryos attach to the endometrium and implant (Heap et al., 1981; Murphy et al., 1981; Murphy et al., 1983).

It is well known that luteal P4, the most common gestagen in mammals, elicits the secretory state of the endometrial glands leading to a favourable intrauterine environment (Spencer et al., 2017). In the pig, conceptus derived estrogens additionally induce the release of secretory vesicles of the endometrial epithelium (Fischer et al., 1985; Geisert et al., 1982a; Roberts et al., 1993). While a direct effect of P4 on bovine embryo elongation *in vitro* could not be observed, elevated peripheral P4 concentrations induced changes of the endometrium that, in turn, accounted for the advancement of embryo elongation (Clemente et al., 2009). In particular, steroid

hormones changed the quality and quantity of uterine secretions (Faulkner et al., 2013; Forde et al., 2009). In marsupials, the resumption of luteal function likely likewise causes endometrial alterations that alter the uterine milieu and determine embryo reactivation (Enders and Given, 1977; Tyndale-Biscoe, 1978).

As in other diapausing species, the resumption of embryonic growth in the roe deer is associated with increased glandular secretions (Aitken, 1975; Aitken et al., 1973). Various studies have shown that plasma P4 remained elevated throughout the period of diapause, where the embryo is in the blastocyst stage and does not elongate (Aitken, 1974b; Sempéré, 1977). Hoffmann et al. (1978) collected monthly plasma samples from 8 pregnant and 3 non-pregnant captive does over the course of one year (Hoffman et al., 1978). According to their observation, plasma P4 levels in pregnant does reached a first peak in August and a second elevation between December and June as compared to non-pregnant does. Total estrogen (E_{tot}) in pregnant animals was lower during August - December compared to January – June. Due to the study design, the corresponding developmental stages of the embryos were unknown and could not be taken into account to discriminate the period around elongation and implantation. A rise in plasma progesterone levels was likewise detected by Sempéré (1977) in captured does in the presumed period of embryo elongation and placentation (January to February, n=21 samples) compared to the presumed period of diapause (October to December, n=25 samples). Unfortunately again, plasma samples were not attributed to more defined developmental stages of the embryo. In contrast, Lambert et al. (2001) analysed plasma P4 concentrations of hunted does where the corresponding embryos were collected by uterine flushing. The plasma P4 levels of the does with blastocysts (n=15), expanded blastocysts (n=3), elongated embryos (n=2) and implanted embryos (n=8) did not differ between developmental stages (Lambert et al., 2001).

Aitken (1974a, 1981) associated embryo elongation with a significant increase in plasma E_{tot}. This finding was supported by Lambert et al. (2001), who reported consistently low estradiol concentrations throughout diapause and expansion, but increased levels at elongation, which remained high at implantation. Although the administration of estrogen to pregnant roe deer during diapause led to an increased blastocyst diameter compared to control animals, it did not lead to elongation (Aitken, 1981).

In ruminants, a placenta cotyledonaria develops after embryo attachment. In this type of placentation, numerous attachment sites, the placentomes, are formed by the maternal caruncular endometrium and the foetal cotyledons. The placenta of many mammalian species is the principle extra-ovarian source of P4 during pregnancy. In ovine, P4 is predominantly produced by the placenta from mid- to late pregnancy, whereas in bovine the CL contributes most (Kindahl et al., 2002). In roe deer, a placental contribution to P4 synthesis has not been investigated yet. Different plasma P4 profiles of pregnant (n=8) and non-pregnant (n=3) animals have been reported, with lower P4 concentrations of non-pregnant animals from January to April (Hoffmann et al., 1978).

Taken together from the results published so far, the contribution of steroid hormones on embryonic growth, reactivation, elongation and placentation in roe deer are inconclusive. The aim of the present study was therefore to investigate the role of P4, E2 and E_{tot} during the roe deer pregnancy in the circulating blood and in the endometrium. For this purpose, we collected samples from 145 does in the course of regular huntings from September to January with known embryonic developmental stages. This period includes the stages of the diapausing blastocyst, embryo elongation and organogenesis.

2. Materials and Methods

2.1. Sample collection

Samples from a total of 145 roe deer does were collected in north-eastern Switzerland and Southern Bavaria between 09/2016-01/2017 and 09/2017-01/2018 during regular, authorized huntings. After a hunt had ended, the shot does were brought to the gathering place for evisceration by the hunters, and the reproductive tracts of female roe deer were removed. Blood was directly retrieved from the heart by cutting through the cranial part of the sternum. The blood was collected in sterile tubes containing EDTA (Monovette, Sarstedt, Germany), and kept on ice until centrifugation at 2000 *g* at 4 °C for 20 minutes. The plasma was stored at -20 °C until further analysis. In some cases, depending on the location where the bullets had penetrated and the way the animals were eviscerated by the hunters, it was not possible to obtain good quality blood because of stomach content and/or faeces contamination. These blood samples were excluded from the study.

Further sample preparation was performed on site in a mobile laboratory van. The reproductive tract was dissected from the mesometrium and the oviducts together with the ovaries were cut off. For the determination of the CL and the follicle number, ovaries were cut open lengthwise. The number and size of the follicles was evaluated under a white light. For embryo collection, the uterus was flushed with 2.5 ml of phosphate buffered saline solution (PBS: pH=7.4; NaCl, $Na_2HPO_42H_2O$, KH_2PO_4, KCl from Merck KGaA and water from Milli-Q® Integral Water Purification System for Ultrapure Water, Merck KGaA). The uterine flushing was immediately evaluated under a stereo microscope (SteREO Discovery Microscope V8, 1:8 Zoom rate, Zeiss) and a photograph of the embryos was taken (Camera Olympus SC50). In case of embryo attachment, the uterus was cut open to recover the conceptus with its membranes. For embryos in the blastocyst stage, the maximal diameter was recorded, while for elongated embryos the maximal length was measured. For embryos in the organogenesis stage, the crown-rump length (CRL) was measured macroscopically with a calliper.

After uterine flushing, the uterus was opened longitudinally and endometrial tissue of the intercaruncular area was carefully collected. The tissue samples were snap frozen in liquid nitrogen and stored at -20 °C until further analysis.

2.2. Determination of steroid hormone concentrations in plasma samples

The hormones P4, E2 and E_{tot} (as simultaneously determined through an antibody cross-reacting to estradiol-17β, estradiol-17α and estrone by 100 %, 70 %, and 100 %, respectively) were determined in plasma as described earlier by Prakash et al. (1987) and Meyer et al. (1990). A competitive enzyme-linked immunosorbent assay (ELISA) was used for the analysis of P4 (antibody as kind courtesy by Franz Weber, LMU Munich, Oberschleissheim, Germany, and 4-pregnen-3,20-dione-3-O-carboxymethyloxime horseradish peroxidase as tracer), E2 (antibody E2/2 Pool 1 and 17 beta-estradiol-6-carboxymethyloxime horseradish peroxidase as tracer) and E_{tot} (antibody E2/3 POOL 1 and estradiol-17-hemisuccinate horseradish peroxidase as tracer) (antibodies kindly provided by Physiology Weihenstephan, Technische Universität München, Germany).

In brief, the steroid hormones were extracted from the plasma with 5 ml tert. butylmethylether/ petrol ether (30/70) (AppliChem, Panraec, ITW Companies), which

was added to 500 µl plasma in an extraction glass prior to analysis. The ELISA was performed in a 96-well microtiter plate reader (Cytation 3 cell imaging multi-mode reader, BioTek). The tracer and antibody dilution, limit of quantification, inter-assay and, intra-assay variation of the different assays are given in Table 1.

Table 1: Enzyme and antibody dilution, limit of quantification, quality control and intra-assay variation of the different hormones

Steroid Hormone	Tracer dilution	Antibody dilution	Limit of quantification	Inter-assay variation	Intra-assay variation
Progesterone	1:11 000	1:210 000	0.09 ng/ml	11 %	7 %
Estradiol-17β	1:30 000	1:350 000	15.9 pg/ml	13 %	13 %
Total estrogens	1:19 000	1:400 000	7.7 pg/ml	14 %	11 %

2.3. Determination of steroid concentrations in endometrial tissue samples

For the analysis of steroid hormone tissue concentrations, approximately 100 mg of intercaruncular endometrium was placed into a plastic tube containing approximately 1000 mg of ceramic beads (MagNa Lyser Green Beads, Roche) and 500 µl of 0.9 % sodium chloride (NaCl; Merck KGaAl) solution. Subsequently, the mixture was homogenized in a MagNa Lyser (Roche) by shaking for 1 min at 7000. Then, each tube was incubated at 4 °C for at least 1 h. The content of each tube was transferred into an extraction glass. P4 and E_{tot} from the tissue mixture were determined following the same protocol as described for extracted plasma.

The E2 ELISA performed poor specifically regarding accuracy. Therefore, endometrial E2 was quantified by radioimmunoassay (RIA). Exemplary plasma samples were measures with RIA and ELISA and showed a good correlation (R= 0.86), while the ELISA overestimated the quantity by 10-fold. However, the absolute concentrations were comparable to those reported earlier, which were determined by the same ELISA (Hoffmann et al., 1978).

For RIA, the dried extracts were re-dissolved in PBS 0.1 % bovine serum albumin and subjected to radioimmunological determination performed by a sequential assay (Strecker et al., 1979) as previously described (Hoffmann et al., 1992; Klein et al., 2003). The antiserum used was directed against E2-6-carboximethyloxim (CMO)–BSA exhibiting a cross-reactivity of 1.3 % for estrone and < 0.01 % for the non-phenolic steroids tested. Intra- and inter-assay CV were 7.1 and 17.6%, respectively. The minimum detectable concentration was 25 pg/g tissue.

2.4. Statistics

To test for the differences of hormone concentrations in plasma and endometrium between different embryonic developmental stages, the animals were grouped according to the size and the developmental stage of the embryo. As stated earlier, it was not possible to sample blood, tissue and the follicle count from each animal. From the 145 does shot in total, we were able to collect blood samples from 106 and endometrial samples from 39 animals.

Animals were assigned to the **blastocyst** group if embryos at the blastocyst stage were recovered (plasma n=62; endometrium n=15-16). Animals were assigned to the **elongation** group if embryos displayed a tubular or filamentous form (plasma n=7; endometrium n=5). Animals were assigned to the **organogenesis** group if the recovered embryos displayed different organ systems, extremities and a head (plasma n=11; endometrium n=5-7).

The log-transformed data of the hormone concentrations were used for statistical analyses using SAS 9.4 (SAS Institute, Inc.,Cary). The data were subjected to least-square analysis of variance using the Mixed Models procedure including the day of sampling as random factor to determine effects of the day of the year and developmental stage of the embryo, respectively. Significant different hormone levels between the developmental stages were assessed by the differences of least-square means with Bonferroni correction. The results from SAS are presented as mean ± SE. Graphs were plotted using GraphPad Prism 7.03 (GraphPad Software).

3. Results

3.1. Corpora lutea and conceptuses recovered

Cyclic does presented between one and three CL. The majority of does (88.7 %) had two CL, while only 4.7 % and 6.6 % presented one and three CL, respectively. The number of embryos recovered from each doe was sometimes less than the number of CL. We obtained zero to three embryos from the uterine flushings per doe. Table 2 shows the the number of CL and the respective number of collected conceptuses.

Table 2: Number of corpora lutea and percentage of the collected conceptuses

Number of corpora lutea	Number of embryos recovered					TOTAL
	0	1	2	3	Unknown*	
1	1 (20 %)	4 (80 %)	-	-	-	5 (100 %)
2	15 (16 %)	22 (23 %)	55 (59 %)	-	2 (2 %)	94 (100 %)
3	2 (28.5%)	-	3 (43 %)	2 (28.5%)	-	7 (100 %)

**The column of "unknown" represents the cases where the uterus was not intact or a part was missing.*

The conceptuses recovered showed different sizes and developmental stages, ranging from blastocyst to elongation, and to organogenesis (Fig. 1). The diameter of the blastocysts ranged from 0.15 to 4.28 mm (1.2 ± 0.73 mm on average). The length of the elongated embryos ranged from 5 mm to several centimetres (25.1 ± 23.0 mm on average). Similarly, embryos at the organogenesis stage showed a large variability in the CRL ranging from 7 to 46 mm (19.0 ± 11.0 mm on average).

3.2. Plasma and endometrial progesterone

The concentration of plasma P4 showed neither a relationship to the number of CL (Fig. 2a), nor to the number of recovered embryos (Fig. 3a) nor to the number of follicles (Suppl. Fig. 1a). The average plasma P4 concentration was 5.3 ± 0.2 ng/ml and ranged between 1.9 and 12.1 ng/ml showing a high inter-individual variance. Plasma P4 was neither affected by the day of sampling (Fig. 4a) nor by the developmental stage (Fig. 4b).

In the endometrial tissue, concentrations of P4 were around ten times higher than in plasma. In contrast to plasma P4, endometrial P4 concentrations differed between the developmental groups (p= 0.0001) (Fig. 5b). Compared to the blastocyst and elongated stage (61.3 ± 5.9 ng/g and 81.4 ± 19.8 ng/g respectively), endometrial P4 concentrations in the organogenesis stage were significantly lower (16.6 ± 4.0 ng/g, $p<0.001$).

The samples derived from does with an embryo in the organogenesis stage were collected late in the sampling period, predominantly between mid/ end of December and February. No correlation was found between day of sampling and endometrial P4 prior organogenesis (Fig. 5a).

3.3. Plasma and endometrial estradiol-17β

The concentration of plasma E2 was neither affected by the number of CL (Fig. 2b), nor by the number of recovered embryos (Fig. 3b) nor by the number of follicles (Suppl. Fig. c). Plasma E2 concentrations showed a vast variability between animals and ranged from 4.3 pg/ml to 135.6 pg/ml with a mean of 25.0 ± 2.5 pg/ml. We observed a high variance between samples collected at the same day (Fig. 4c), and between samples corresponding to the same developmental stage, especially in the animals with blastocysts smaller than 2 mm and during organogenesis. Taking the sampling date into account, a decline of plasma E2 from the blastocyst group (27.0 ± 3.4 pg/ml) to the group with elongated embryos (17.5 ± 7.4 pg/ml) was detected (p= 0.017). In the organogenesis group, the average plasma E2 concentration was higher compared to the blastocyst and elongated stage (35.0 ± 11.6 pg/ml). Nevertheless, when only taking the developmental stage into account, the difference between the groups was not significant (Fig. 4d). A decline of both the number of follicles and plasma E2 was observed over time.

Intercaruncular endometrial E2 was around ten-fold higher than plasma E2. In the endometrium, E2 ranged from 92.6 to 483.7 pg/g. In contrast to plasma E2, a gradual decline of endometrial E2 from the blastocyst (302.4 ± 28.3 pg/g) to the organogenesis stage (150.0 ± 24.4 pg/g) was evident (Fig. 5d). This gradual decrease was also observed when relating either the date of sampling (Fig. 5c) or the size of the conceptuses to endometrial E2 concentrations. The differences of endometrial E2 concentrations between the developmental stages was significant (p= 0.047).

3.4. Plasma and endometrial total estrogens

The concentration of plasma E_{tot} was neither affected by the number of CL (Fig. 2c), nor by the number of recovered embryos (Fig. 3c) nor by the number of follicles (Suppl. Fig. e). The pattern of E_{tot} related to the date of sampling, the size of the conceptus and the number of follicles was similar to the one of E2. E_{tot} concentrations presented a high variability over time (Fig. 4e), which declined from smaller embryos to longer ones. There was no difference of E_{tot} between the blastocyst (21.7 ± 2.9 pg/ml), the elongated (31.4 ± 17.7 pg/ml) and the organogenesis group (34.4 ± 15.4 pg/ml) (Fig. 4f).

Numerically, endometrial concentrations of E_{tot} were around 200 times higher than in plasma. In contrast to the endometrial E2, the mean values of E_{tot} in this tissue stayed constant over the embryonic period of blastocyst and elongation (Fig. 5f) with a mean value of 443.8 ± 52.4 pg/g and 420.0 ± 49.0 pg/g, respectively. During organogenesis, values were slightly higher (585.7 ± 155.0 pg/g). There were no changes in E_{tot} over the sampling period (Fig. 5e). Nevertheless, when comparing with the conceptus size, the variability of endometrial E_{tot} values from embryos of the blastocyst stage with a diameter of until 3 mm was higher than the values thereafter.

3.5. Number of follicles

The mean number of follicles per doe was 21.5 follicles (of n=45 does), where the lowest observed number was 3 and the maximum 51 follicles per ovary. When comparing the number of follicles over time (Fig. 6a), and taking into account the developmental stage of the conceptuses, we found significant differences between the three developmental groups (p= 0.0011). Similar to E2, the number of follicles declined from the blastocyst to the elongated embryo group. At the stage of organogenesis around late December-January, the follicle count increased again. Nevertheless, when

we compared the developmental stages between themselves (Fig. 6b) no differences were found.

4. Discussion

We did not detect any difference in the concentrations of neither plasma nor endometrial P4, E2 and E_{tot} from the blastocyst to the elongated embryo stage. We therefore conclude that the constant concentration of P4 during diapause provides a progestational milieu for the blastocyst enabling slow but continuous growth. Thereafter, the resumption of normal growth velocity that is associated with embryo elongation is independent from a change in either P4, E2 or E_{tot}. Maternal support of escape from diapause in roe deer, if necessary at all, is thus not directly driven by ovarian P4.

Likewise, we did not observe the sampling date affecting P4 concentrations, which renders a photoperiod driven P4 stimulation of embryonic growth velocity via the hypothalamic-pituitary-ovarian-axis unlikely. Our findings are therefore in contrast to the control of embryo activation after diapause in mustelids and marsupials, which is regulated by photoperiod-induced changes of luteal function (Heap et al., 1981; Murphy et al., 1981; Murphy et al., 1983). In the tammar wallaby (Hinds and den Ottolander, 1983; Sadleir and Tyndale-Biscoe, 1977), the mink (Allais and Martinet, 1978) and the badger (Canivenc and Bonnin, 1981; Nobuyuki Yamaguchi et al., 2006) the period of diapause was shortened due to a change in photoperiod, concomitant with a highly synchronized birth. Here, the diapausing embryo is maintained in a milieu obviously not suitable for further development. P4 acting on the endometrium is then able to term embryonic growth arrest (Lefèvre et al., 2011; Lopes et al., 2006; Lopes et al., 2003; Martin et al., 2016; Murphy, 2012; Renfree and Shaw, 2014). The assumption that photoperiod does not drive embryonic growth velocity in roe deer is further supported by the great variability of developmental stages encountered around the presumed period of growth resumption at the time of winter solstice on December 21st. Our observations match the preliminary findings of Lincoln and Guinness (1972). These authors tested in an experimental approach if the exposure to an artificial light regime that mimicked shorter day length would reduce the period of diapause in roe deer and result in birth prior to the physiological fawning season. For that purpose, two does with observed mating were enclosed in a lightproof shed at defined times each day, thereby progressively reducing the exposure to natural light from beginning to the

end of October. Thereafter, increasing daylight length was generated by exposure to artificial light until beginning of December. Of the two does, only one proved to be pregnant and gave birth during the natural fawning season mid of May, indicating that the period of reduced growth had not shortened in response to the artificially advanced winter solstice (Lincoln and Guinness, 1972).

In our study, the number of CL explained neither plasma nor endometrial P4 concentrations. Thus, there may be a threshold P4 concentration, being around >1 ng/ml in plasma, for maintaining pregnancy. We observed a high individual variation in plasma and endometrial estrogen concentrations being most pronounced at the blastocyst stage. A local temporary rise of endometrial estrogens might thus still underlay the present data. As in bovine, the estrogen synthesized by luteal cells during the luteal phase (Okuda et al., 2001) could act locally in the endometrium without being elevated in circulating plasma. However, the number of CL explained neither plasma E2 nor E_{tot}. From our data, neither the number of follicles could explain plasma estrogens. Most follicles were present during presence of a blastocyst staged embryo, mainly corresponding to the sampling period between November and December. During this time, the ovaries exhibited more follicles than later in the season.

The assumption that the granulosa cells of the follicles are the main source of estrogens (Moon et al., 1978), could explain our findings of significantly lower endometrial E2 between the blastocyst and elongated stage on the one hand and the stage of organogenesis on the other, which was reached in December and January. Unfortunately, this remains hypothetical as the status of the follicles (growing or atretic) was not accessed.

Interestingly, the decrease of endometrial E2 between the blastocyst and elongated stage, respectively, and the stage of organogenesis was not evident in plasma. Although not statistically significant, endometrial E_{tot} concentrations were higher during organogenesis than at earlier stages. The reason could be a shift from endometrial E2 synthesis to estrone production that would be reflected in E_{tot}. While in ruminants there is no evidence of endometrial production of steroid hormones to date (Mann et al., 2007), the pig endometrium is capable of oestrogen production at the time of pregnancy recognition (Franczak, 2008; Mann et al., 2007). Another possible source of estrone production is the embryo itself. In fact, *in vitro* studies of Gadsby et al. (1980) showed that the pig trophoblast and the roe deer allantochorion produced estrone only

after attachment, which was not the case for earlier, not yet attached embryo stages. Thus, estrone produced by the elongated allantochorionic sacs could accumulate in the apposed maternal endometrium and account for the continuous high concentrations of our study. The cotyledones are likewise another possible source of estrogen synthesis (Schuler et al., 2006; Schuler et al., 2002).

We observed 10- to 20-fold higher steroid concentrations in the reproductive tissues than in the plasma. Similar observations have been reported in bovine (Mann et al., 2007) and ovine (Abecia et al., 1996) and can probably be attributed to different matrix properties. In addition to a the local presence of steroid hormone receptors, the direct blood supply by the ovarian vein and the uterine artery could greatly increase the concentration of circulating ovarian steroid hormones in the reproductive tract (Weems et al., 1989; Weems et al., 1988).

At the stage of organogenesis, P4 concentration showed a clear decline in the intercaruncular endometrium compared to the blastocyst and elongated stage. The caruncular endometrium, together with the embryonic cotyledons, forms the multiple placentomes characteristic for ruminant placentation. In the bovine, the placentomes are known to contribute to the obligatory luteal P4 production (Hoffmann and Schuler, 2002). In the white tailed deer, pregnancy is also dependent on luteal P4 production as lutectomy in the second half of pregnancy leads to abortion (Plotka et al., 1982). In the roe deer, the production of P4 by the placentomes has yet to be determined. Therefore, this finding remains puzzling.

5. Conclusions

In summary, the results obtained from the endometrial as well as from the plasma analyses do not show any indication that changes in ovarian steroid hormones trigger embryonic reactivation and thus the termination of diapause in the roe deer. Further investigations are necessary to explore the factors controlling the end of the diapause and their sources.

6. Declaration of interest

All authors confirm that there is no conflict of interest interfering with the impartiality of the scientific work.

7. Funding

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9. References

Abecia, J.A., Rhind, S.M., Goddard, P.J., McMillen, S.R., Ahmadi, S., Elston, D.A., 1996. Jugular and ovarian venous profiles of progesterone and associated endometrial progesterone concentrations in pregnant and non-pregnant ewes. Animal Science 63, 229-234.

Aitken, R.J., 1974a. Delayed implantation in roe deer (Capreolus capreolus). Journal of Reproduction and Fertility 39, 225-233.

Aitken, R.J., 1974b. Delayed Implantation in the roe deer (Capreolus capreolus).

Aitken, R.J., 1975. Ultrastructure of the blastocyst and endometrium of the roe deer (Capreolus capreolus) during delayed implantation. Journal of anatomy 119, 369-384.

Aitken, R.J., 1981. Aspects of delayed implantation in the roe deer (Capreolus capreolus). Journal of Reproduction and Fertility. Supplement 29, 83-95.

Aitken, R.J., Burton, J., Hawkins, J., Kerr-Wilson, R., S.R.V., Steven, D.H., 1973. Histological and ultrastructural changes in the blastocyst and reproductive tract of the roe deer, capreolus capreolus, during delayed implantation. Journal of Reproduction and Fertility 34, 481-493.

Allais, C., Martinet, L., 1978. Relation between daylight ratio, plasma progesterone levels and timing of nidation in mink (Mustela vison). Journal of Reproduction and Fertility 54, 133-136.

Bazer, F.W., 2013. Pregnancy recognition signaling mechanisms in ruminants and pigs. Journal of animal science and biotechnology 4, 23.

Bazer, F.W., Thatcher, W.W., 1977. Theory of maternal recognition of pregnancy in swine based on estrogen controlled endocrine versus exocrine secretion of prostaglandin F2α by the uterine endometrium. Prostaglandins 14, 397-401.

Bischoff, T.L.W., 1854. Entwicklungsgeschichte des Rehes. Rickersche Buchhandlung, Gießen.

Canivenc, R., Bonnin, M., 1981. Environmental control of delayed implantation in the European badger (Meles meles). Journal of reproduction and fertility. Supplement 29, 25-33.

Clemente, M., de La Fuente, J., Fair, T., Al Naib, A., Gutierrez-Adan, A., Roche, J.F., Rizos, D., Lonergan, P., 2009. Progesterone and conceptus elongation in cattle: a direct effect on the embryo or an indirect effect via the endometrium? Reproduction 138, 507-517.

Enders, A.C., Given, R.L., 1977. The Endometrium of Delayed and Early Implantation, in: Wynn, R.M. (Ed.), Biology of the Uterus. Springer US, Boston, MA, pp. 203-243.

Faulkner, S., Elia, G., O'Boyle, P., Dunn, M., Morris, D., 2013. Composition of the bovine uterine proteome is associated with stage of cycle and concentration of systemic progesterone. Proteomics 13, 3333-3353.

Finn, C.A., Martin, L., 1972. Endocrine Control of the Timing of Endometrial Sensitivity to a Decidual Stimulus. Biology of Reproduction 7, 82-86.

Fischer, H.E., Bazer, F.W., Fields, M.J., 1985. Steroid metabolism by endometrial and conceptus tissues during early pregnancy and pseudopregnancy in gilts. Journal of Reproduction and Fertility 75, 69-78.

Flint, A.P., Krzywinski, A., Sempere, A.J., Mauget, R., Lacroix, A., 1994. Luteal oxytocin and monoestry in the roe deer Capreolus capreolus. J Reprod Fertil 101, 651-656.

Flint, A.P.F., Heap, R.B., Gadsby, J.E., Saunders, P.T.K., 1979. Blastocyst oestrogen synthesis and the maternal recognition of pregnancy, in: Maternal Recognition of Pregnancy. (J. Whelan, ed.), Ciba Foundation Colloquium, Excerpta Medica, Amsterdam. 64, 209–228.

Forde, N., Carter, F., Fair, T., Crowe, M.A., Evans, A.C.O., Spencer, T.E., Bazer, F.W., McBride, R., Boland, M.P., O'Gaora, P., Lonergan, P., Roche, J.F., 2009. Progesterone-Regulated Changes in Endometrial Gene Expression Contribute to Advanced Conceptus Development in Cattle. Biology of Reproduction 81, 784-794.

Franczak, A., 2008. Endometrial and myometrial secretion of androgens and estrone during early pregnancy and luteolysis in pigs. Reproductive Biology 8, 213-228.

Gadsby, J.E., Heap, R.B., Burton, R.D., 1980. Oestrogen production by blastocyst and early embryonic tissue of various species. Journal of Reproduction and Fertility 60, 409-417.

Gandolfi, F., Brevini, T.A.L., Modina, S., Passoni, L., 1992. Early embryonic signals: embryo-maternal interactions before implantation. Anim Reprod Sci 28, 269-276.

Geisert, R.D., Brookbank, J.W., Michael Roberts, R., Bazer, F.W., 1982a. Establishment of Pregnancy in the Pig: II. Cellular Remodeling of the Porcine Blastocyst During Elongation on Day 12 of Pregnancy12. Biology of Reproduction 27, 941-955.

Geisert, R.D., Renegar, R.H., Thatcher, W.W., Roberts, R.M., Bazer, F.W., 1982b. Establishment of pregnancy in the pig: I. Interrelationships between preimplantation development of the pig blastocyst and uterine endometrial secretions. Biology of reproduction 27, 925-939.

Heap, R.B., Flint, A.P.F., Gadsby, J.E., 1981. Embryonic Signals and Maternal Recognition, in: Glasser, S.R., Bullock, D.W. (Eds.), Cellular and Molecular Aspects of Implantation. Springer US, Boston, MA, pp. 311-326.

Hinds, L.A., den Ottolander, R.C., 1983. Effect of changing photoperiod on peripheral plasma prolactin and progesterone concentrations in the tammar wallaby (Macropus eugenii). Journal of Reproduction and Fertility 69, 631-639.

Hoffmann, B., Barth, D., Karg, H., 1978. Progesterone and estrogen levels in peripheral plasma of the pregnant and nonpregnant roe deer (Capreolus capreolus). Biology of Reproduction 19, 931-935.

Hoffmann, B., Höveler, R., Hasan, S., Failing, K., 1992. Ovarian and pituitary function in dogs after hysterectomy. Journal of reproduction and fertility 96, 837-845.

Hoffmann, B., Schuler, G., 2002. The bovine placenta; a source and target of steroid hormones: observations during the second half of gestation. Domestic Animal Endocrinology 23, 309-320.

Kindahl, H., Kornmatitsuk, B., Königsson, K., Gustafsson, H., 2002. Endocrine changes in late bovine pregnancy with special emphasis on fetal well-being. Domestic animal endocrinology 23, 321-328.

Klein, R., Schams, D., Failing, K., Hoffmann, B., 2003. Investigations on the Re-establishment of the Positive Feedback of Oestradiol during Anoestrus in the Bitch. Reproduction in domestic animals 38, 13-20.

Lambert, R.T., Ashworth, C.J., Beattie, L., Gebbie, F.E., Hutchinson, J.S., Kyle, D.J., Racey, P.A., 2001. Temporal changes in reproductive hormones and conceptus-endometrial interactions during embryonic diapause and reactivation of the blastocyst in European roe deer (Capreolus capreolus). Reproduction 121, 863-871.

Lefèvre, P.L.C., Palin, M.-F., Beaudry, D., Dobias-Goff, M., Desmarais, J.A., Llerena V., E.M., Murphy, B.D., 2011. Uterine signaling at the emergence of the embryo from obligate diapause. American Journal of Physiology - Endocrinology And Metabolism 300, E800-E808.

Lengwinat, T., Meyer, H.H., 1996. Investigations of BrdU incorporation in roe deer blastocysts in vitro. Anim Reprod Sci 45, 103-107.

Lincoln, G.A., Guinness, F.E., 1972. Effect of altered photoperiod on delayed implantation and moulting in roe deer. Journal of Reproduction and Fertility 31, 455-457.

Lopes, F.L., Desmarais, J., Ledoux, S., Gévry, N.Y., Lefevre, P., Murphy, B.D., 2006. Transcriptional Regulation of Uterine Vascular Endothelial Growth Factor during Early Gestation in a Carnivore Model, Mustela vison. Journal of Biological Chemistry 281, 24602-24611.

Lopes, F.L., Desmarais, J.A., Murphy, B.D., 2004. Embryonic diapause and its regulation. Reproduction 128, 669-678.

Lopes, F.L., Desmarais, J.I.A., Gévry, N.Y., Ledoux, S., Murphy, B.D., 2003. Expression of Vascular Endothelial Growth Factor Isoforms and Receptors Flt-1 and KDR During the Peri-Implantation Period in the Mink, Mustela vison1. Biology of Reproduction 68, 1926-1933.

Mann, G.E., Scholey, D.V., Robinson, R.S., 2007. Identification of elevated concentrations of estradiol in bovine uterine endometrium. Domestic animal endocrinology 33, 437-441.

Martin, F.C., Ang, C.-S., Gardner, D.K., Renfree, M.B., Shaw, G., 2016. Uterine flushing proteome of the tammar wallaby after reactivation from diapause. Reproduction 152, 491-505.

Mead, R.A., 1989. The Physiology and Evolution of Delayed Implantation in Carnivores, in: Gittleman, J.L. (Ed.), Carnivore Behavior, Ecology, and Evolution. Springer US, Boston, MA, pp. 437-464.

Mead, R.A., 1993. Embryonic diapause in vertebrates. Journal of Experimental Zoology 266, 629-641.

Meyer, H.H.D., Sauerwein, H., Mutayoba, B.M., 1990. Immunoaffinity chromatography and a biotin-streptavidin amplified enzymeimmunoassay for sensitive and specific estimation of estradiol-17β. Journal of Steroid Biochemistry 35, 263-269.

Moon, Y.S., Tsang, B.K., Simpson, C., Armstrong, D.T., 1978. 17β-Estradiol Biosynthesis in Cultured Granulosa and Thecal Cells of Human Ovarian Follicles: Stimulation by Follicle-Stimulating Hormone*. The Journal of Clinical Endocrinology & Metabolism 47, 263-267.

Murphy, B.D., 2012. Embryonic Diapause: Advances in Understanding the Enigma of Seasonal Delayed Implantation. Reproduction in Domestic Animals 47, 121-124.

Murphy, B.D., Concannon, P.W., Travis, H.F., Hansel, W., 1981. Prolactin: The Hypophyseal Factor That Terminates Embryonic Diapause in Mink. Biology of Reproduction 25, 487-491.

Murphy, B.D., Mead, R.A., McKibbin, P.E., 1983. Luteal Contribution to the Termination of Preimplantation Delay in Mink. Biology of Reproduction 28, 497-503.

Nobuyuki Yamaguchi, Hannah L Dugdale, David W Macdonald, 2006. Female Receptivity, Embryonic Diapause, and Superfetation in the European Badger (Meles meles): Implications for the Reproductive Tactics of Males and Females. The Quarterly Review of Biology 81, 33-48.

Okuda, K., Uenoyama, Y., Berisha, B., Lange, I.G., Taniguchi, H., Kobayashi, S., Kobayashi, S.-i., Miyamoto, A., Schams, D., 2001. Estradiol-17β Is Produced in Bovine Corpus Luteum. Biology of Reproduction 65, 1634-1639.

Plotka, E.D., Seal, U.S., Verme, L.J., Ozoga, J.J., 1982. Reproductive Steroids in White-Tailed Deer. IV. Origin of Progesterone During Pregnancy1. Biology of Reproduction 26, 258-262.

Prakash, B.S., Meyer, H.H.D., Schallenberger, E., van De Wiel, D.F.M., 1987. Development of a sensitive enzymeimmunoassay (EIA) for progesterone determination in unextracted bovine plasma using the second antibody technique. Journal of Steroid Biochemistry 28, 623-627.

Renfree, M.B., 1981. Embryonic diapause in marsupials. Journal of reproduction and fertility. Supplement 29, 67-78.

Renfree, M.B., Shaw, G., 2014. Embryo-endometrial interactions during early development after embryonic diapause in the marsupial tammar wallaby. Int J Dev Biol 58, 175-181.

Roberts, R.M., Xie, S., Trout, W.E., 1993. Embryo-uterine interactions in pigs during week 2 of pregnancy. Journal of reproduction and fertility. Supplement 48, 171-186.

Sadleir, R.M.F.S., Tyndale-Biscoe, C.H., 1977. Photoperiod and the Termination of Embryonic Diapause in the Marsupial Macropus eugenii. Biology of Reproduction 16, 605-608.

Schams, D., Barth, D., Karg, H., 1980. LH, FSH and progesterone concentrations in peripheral plasma of the female roe deer (capreolus capreolus) during the rutting season. J. Reprod. Fert 60, 109-114.

Schuler, G., Özalp, G.R., Hoffmann, B., Harada, N., Browne, P., Conley, A.J., 2006. Reciprocal expression of 17α-hydroxylase-C17,20-lyase and aromatase cytochrome P450 during bovine trophoblast differentiation: a two-cell system drives placental oestrogen synthesis. Reproduction 131, 669-679.

Schuler, G., Wirth, C., Teichmann, U., Failing, K., Leiser, R., Thole, H., Hoffmann, B., 2002. Occurrence of Estrogen Receptor α in Bovine Placentomes Throughout Mid and Late Gestation and at Parturition1. Biology of Reproduction 66, 976-982.

Sempéré, A.J., 1977. Plasma Progesterone Levels in Roe Deer, Capreolus-Capreolus. Journal of Reproduction and Fertility 50, 365-366.

Short, R.V., Hay, M.F., 1966. Delayed implantation in the roe deer (*Capreolus capreolus*), Symposium of the Zoological Society of London, pp. 173-194.

Spencer, T.E., Forde, N., Lonergan, P., 2017. Insights into conceptus elongation and establishment of pregnancy in ruminants. Reproduction, Fertility and Development 29, 84-100.

Sponchiado, M., Gomes, N.S., Fontes, P.K., Martins, T., del Collado, M., Pastore, A.d.A., Pugliesi, G., Nogueira, M.F.G., Binelli, M., 2017. Pre-hatching embryo-dependent and -independent programming of endometrial function in cattle. PLOS ONE 12, e0175954.

Strecker, H., Hachmann, H., Seidel, L., 1979. Der Radioimmunotest (RIA), eine hochspezifische, extrem empfindliche quantitative Analysenmethode. Chemiker Zeitung 103, 53-68.

Tyndale-Biscoe, C.H., 1978. Hormonal control of embryonic diapause and reactivation in the tammar wallaby. Ciba Foundation symposium, 173-190.

Weems, C., Weems, Y., Lee, C., Vincent, D., 1989. Progesterone in uterine and arterial tissue and in jugular and uterine venous plasma of sheep. Biology of reproduction 41, 1-6.

Weems, C.W., Lee, C.N., Weems, Y.S., Vincent, D.L., 1988. Distribution of Progesterone to the Uterus and Associated Vasculature of Cattle. Endocrinologia Japonica 35, 625-630.

Young, S.L., 2013. Oestrogen and progesterone action on endometrium: a translational approach to understanding endometrial receptivity. Reproductive biomedicine online 27, 497-505.

Ziegler, 1843. Beobachtungen über die Brunst und den Embryo der Rehe. Hellweg'sche Hofbuchhandlung, Hannover.

10. Figure Legends

Figure 1: Embryo sizes recovered during the sampling period are grouped according to the following three developmental stages: (A) embryos at the blastocysts stage (**Blastocysts**) and embryos at the elongated blastocysts stage (**Elongated Embryos**), and (B) embryos undergoing organogenesis (**Organogenesis**). CRL - crown-rump-length during organogenesis.

Figure 2: Plasma concentration of (A) P4, (B) E2 and (C) E_{tot} related to the number of CL. Mean values are represented by lines. There was no significant effect of the number of CL on any plasma steroid hormone ($p > 0.05$).

Figure 3: Plasma concentration of (A) P4, (B) E2 and (C) E_{tot} and the number of embryos recovered. Mean values are represented by lines. There was no significant effect of the number of embryos on plasma steroid hormone nor endometrial steroid hormone concentration ($p > 0.05$).

Figure 4: Plasma hormone concentrations during the period of diapause and ongoing embryo development (A, C and E) and at defined developmental stages (B, D and F). Mean values are represented by lines. There was no significant effect of neither the date of sampling nor the developmental stage on plasma hormone concentration ($p > 0.05$).

Figure 5: Endometrial tissue hormone concentrations during the period of diapause and ongoing embryo development (A, C and E) and at defined developmental stages (B, D and F). Mean values are represented by lines. Significant differences between the groups are indicated by * ($p = 0.05$) and ** ($p < 0.001$).

Figure 6: Number of follicles on both ovaries during the period of diapause and ongoing embryo development (A) and at defined developmental stage (B). Mean values are represented by lines. There was no significant effect of neither the date of sampling nor the developmental stage on the number of follicles ($p > 0.05$).

Supplementary Figure: Steroid hormone concentration in plasma (A, C and E) and endometrial tissue (B, D and F) related to the number of follicles on both ovaries. There was no significant effect of the number of follicles on neither plasma nor endometrial steroid hormone concentration ($p > 0.05$).

11. Figures

Figure 1

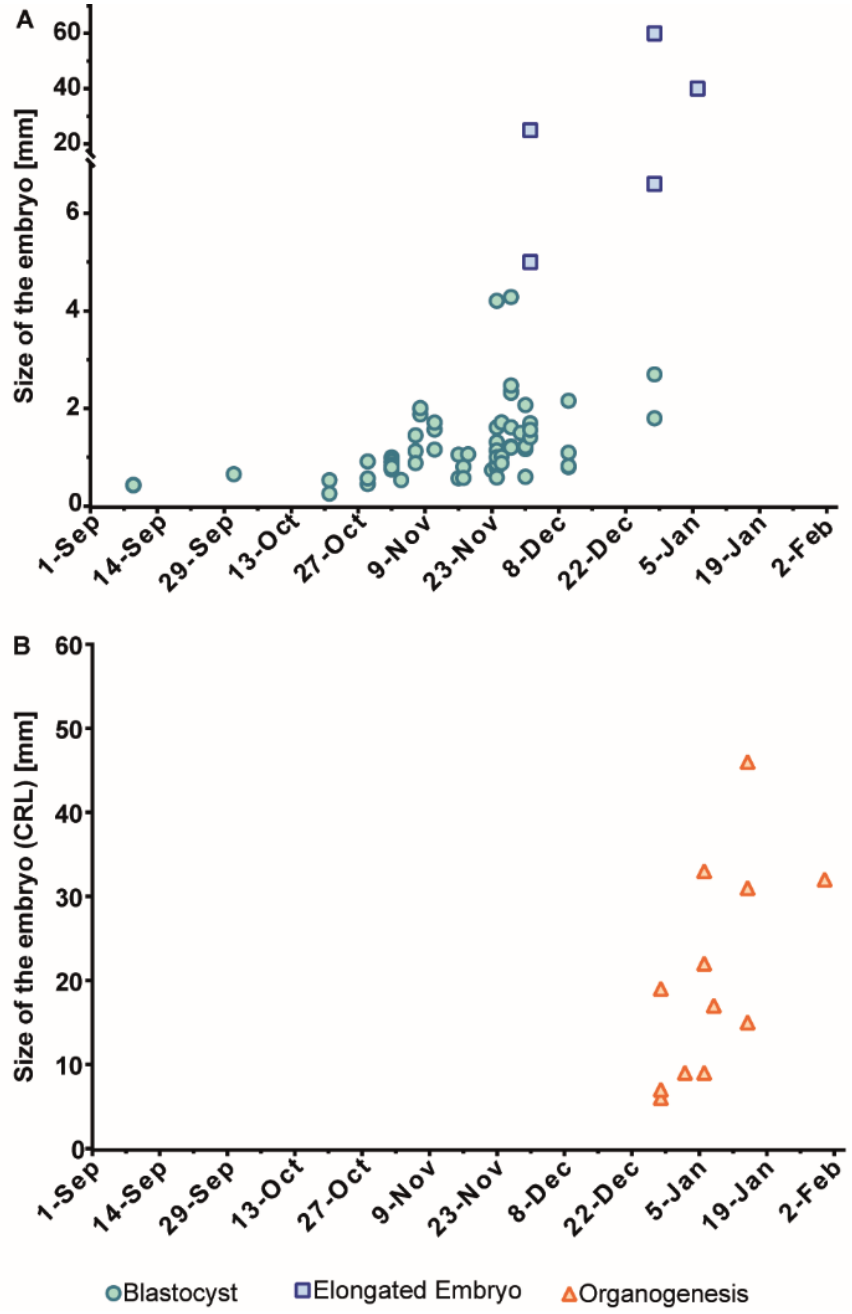


Figure 2

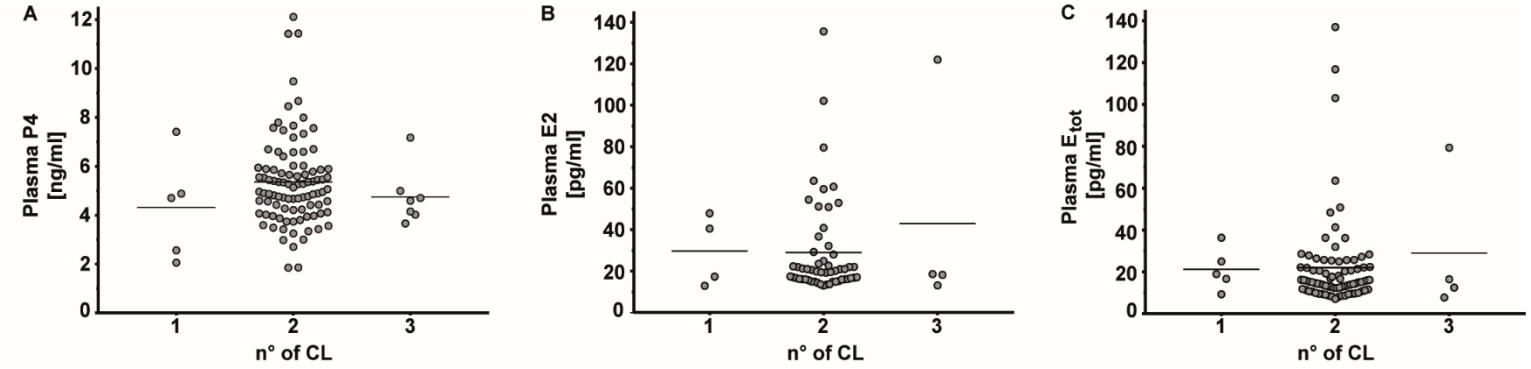


Figure 3

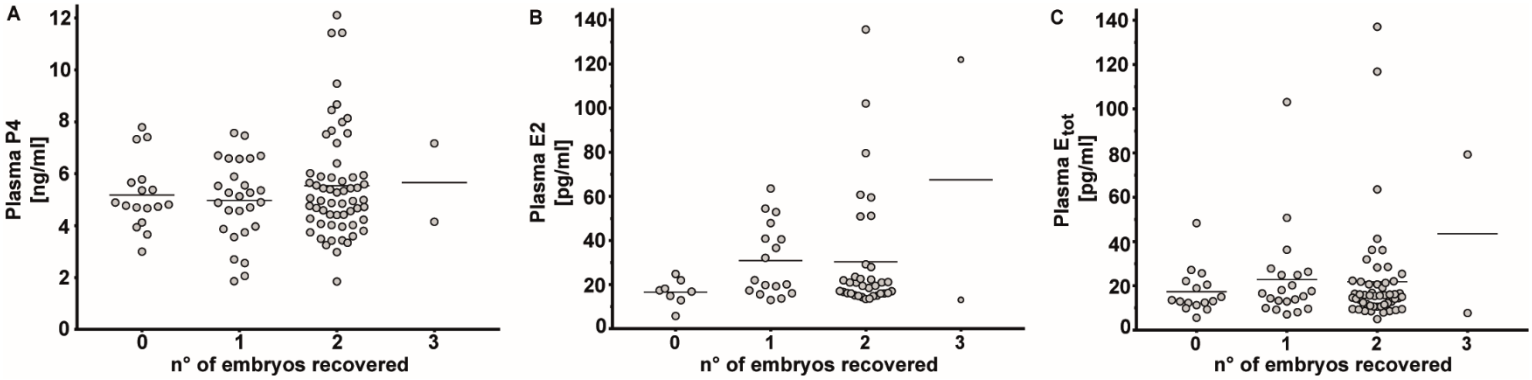


Figure 4

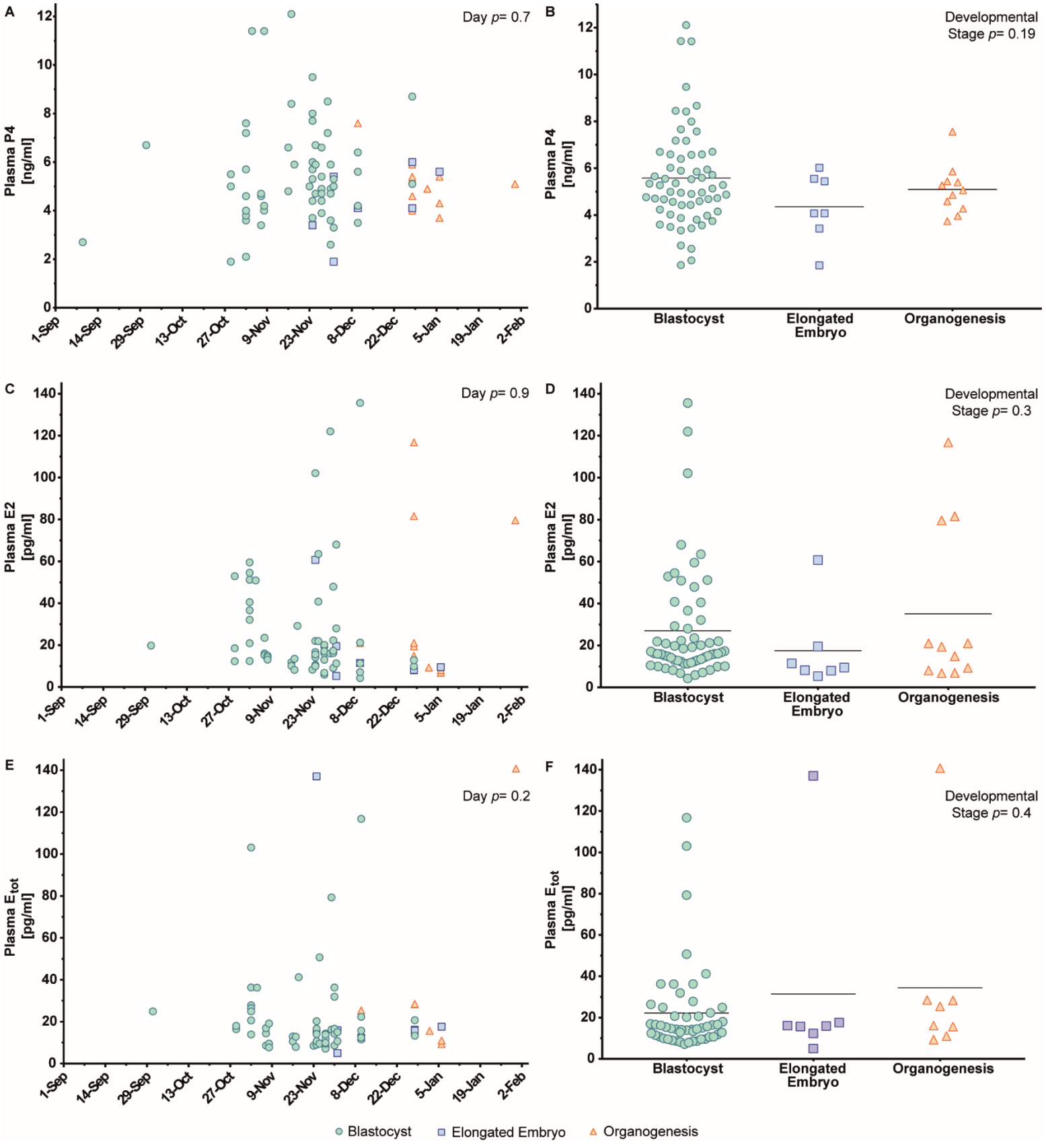

A
Day p= 0.7
Plasma P4 [ng/ml]
1-Sep
14-Sep
29-Sep
13-Oct
27-Oct
9-Nov
23-Nov
8-Dec
22-Dec
5-Jan
19-Jan
2-Feb
B
Developmental Stage p= 0.19
Plasma P4 [ng/ml]
Blastocyst
Elongated Embryo
Organogenesis
C
Day p= 0.9
Plasma E2 [pg/ml]
D
Developmental Stage p= 0.3
Plasma E2 [pg/ml]
E
Day p= 0.2
Plasma Etot [pg/ml]
F
Developmental Stage p= 0.4
Plasma Etot [pg/ml]
Blastocyst
Elongated Embryo
Organogenesis

Figure 5

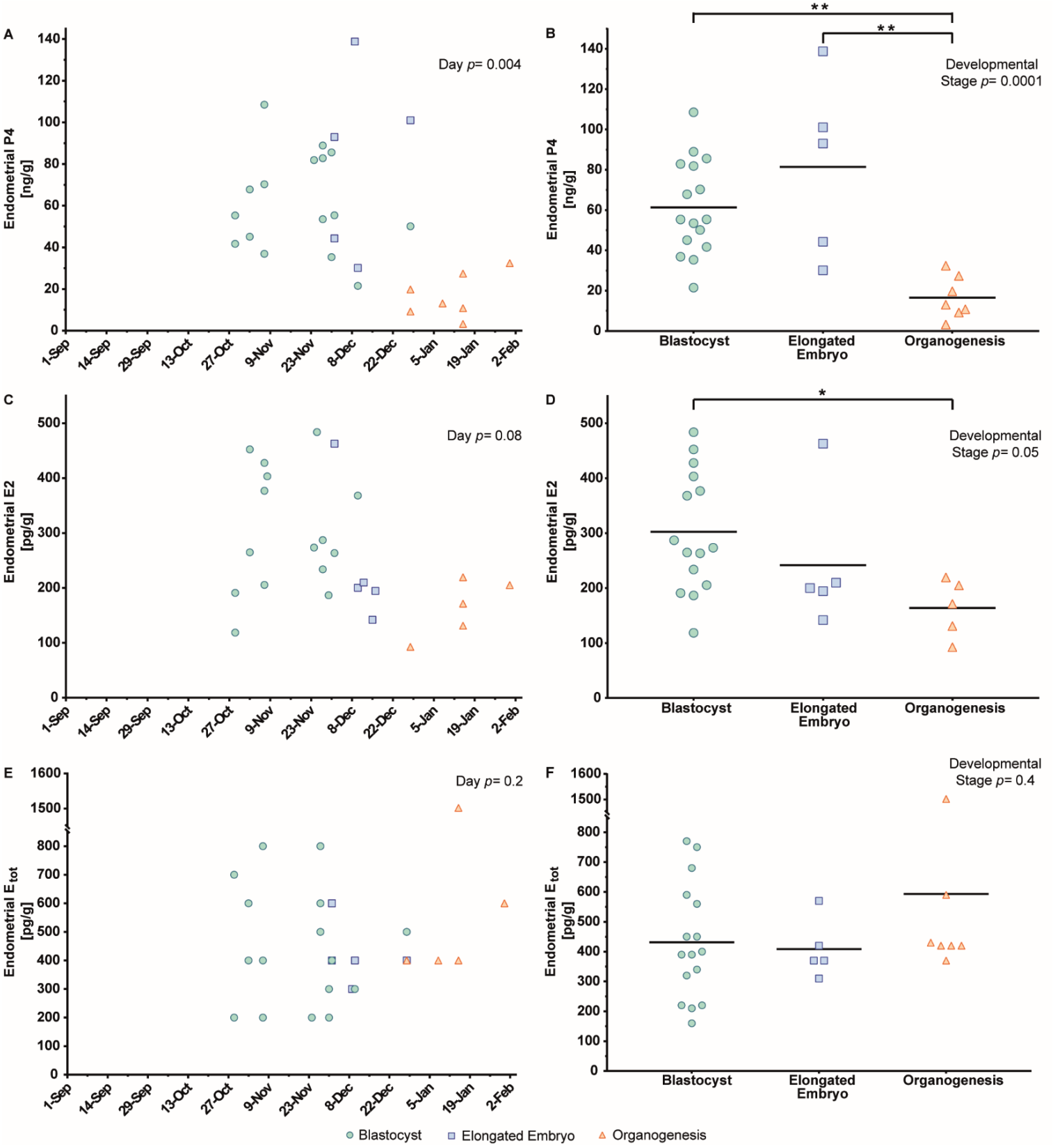
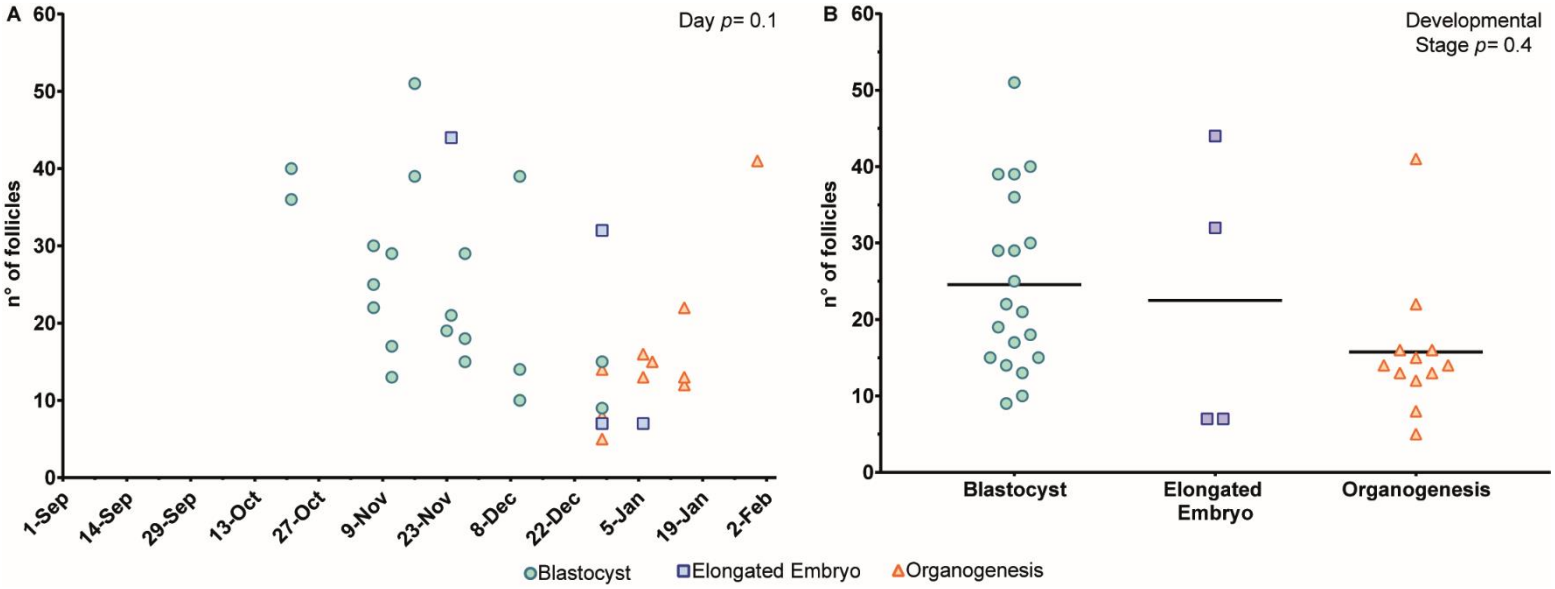
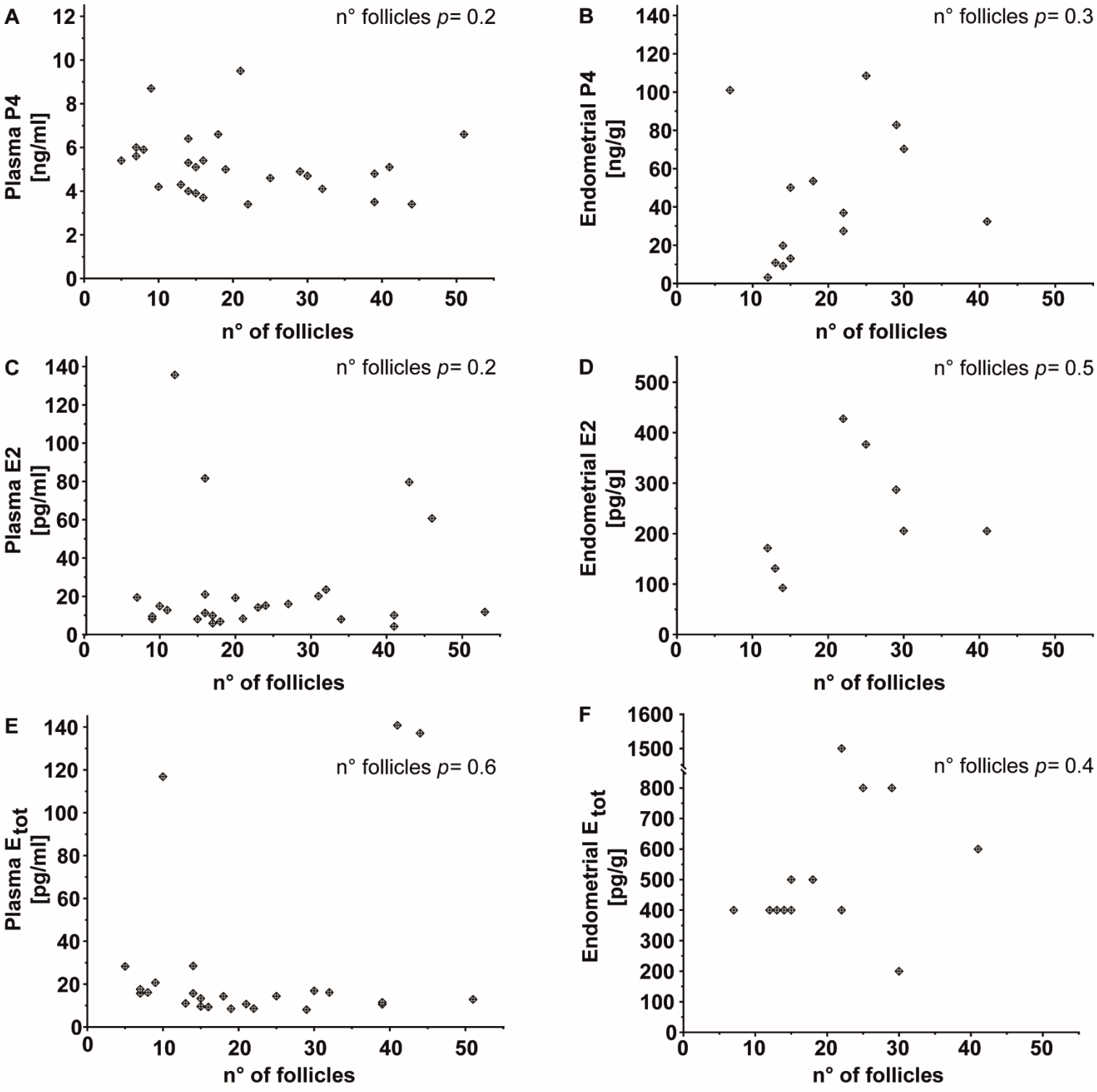

A
Endometrial P4 [ng/g]
Day p= 0.004
1-Sep
14-Sep
29-Sep
13-Oct
27-Oct
9-Nov
23-Nov
8-Dec
22-Dec
5-Jan
19-Jan
2-Feb
B
Developmental Stage p= 0.0001
**
**
Blastocyst
Elongated Embryo
Organogenesis
C
Endometrial E2 [pg/g]
Day p= 0.08
D
Developmental Stage p= 0.05
*
E
Endometrial E tot [pg/g]
Day p= 0.2
F
Developmental Stage p= 0.4
Blastocyst
Elongated Embryo
Organogenesis

Figure 6


A
B
n° of follicles
Day p= 0.1
Developmental Stage p= 0.4
1-Sep
14-Sep
29-Sep
13-Oct
27-Oct
9-Nov
23-Nov
8-Dec
22-Dec
5-Jan
19-Jan
2-Feb
Blastocyst
Elongated Embryo
Organogenesis

Supplementary Figure



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